GENETIC DIFFERENCES BETWEEN TUNISIAN CAMEL AND SHEEP STRAINS OF THE CESTODE *ECHINOCOCCUS GRANULOSUS* REVEALED BY SSCP

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Summary:

Ovine and dromedary *Echinococcus granulosus* isolates from Tunisia were identified as G1 and G6 strains based on polymorphism of the mitochondrial cytochrome C oxydase CO1. Single strand conformation polymorphism (SSCP) was used in order to examine the genetic variation within and between Tunisian G1 and G6 strains and to estimate the extent of selfing. The dromedary isolates are genetically distinct from sheep isolates (high value of genetic variation between populations: $F_{st} = 0.46$). No significant deficiency in heterozygotes was found in sheep isolates, whereas heterozygote deficiency (suggesting selfing) was found in a limited number of camel isolates.

KEY WORDS : *Echinococcus granulosus*, hydatidosis, SSCP, genetic variation, strain, Tunisia.

Résumé : DIFFÉRENCES GÉNÉTIQUES ENTRE LES LIGNÉES TUNISIENNES OVINE ET CAMELINE DU CESTODE *ECHINOCOCCUS GRANULOSUS* FONDÉES SUR LA SSCP

Les Echinococcus granulosus *de Tunisie ont été identifiés comme appartenant aux lignées G1 et G6 en se fondant sur le polymorphisme de la cytochrome oxydase CO1. Le polymorphisme de conformation simple brin (SSCP) a été utilisé afin d'évaluer la variabilité génétique intra et inter-isolats des lignées G1 et G6, et pour estimer le taux d'autofécondation. Les isolats issus de dromadaires sont génétiquement distincts des isolats ovins (valeur de F_{st} : 0,46). Il n'y a pas de déficit significatif en hétérozygotes chez les isolats ovins. Un déficit en hétérozygotes est présent dans les isolats du dromadaire, ce qui suggère l'autofécondation comme l'un des modes de reproduction.*

MOTS CLÉS : Echinococcus granulosus, *hydatidose, polymorphisme de conformation simple brin, variabilité génétique, lignée, Tunisie.*

INTRODUCTION

The cystic echinococcosis is a major public health problem throughout the world. In Tunisia, the disease leads to serious socio-economic repercussions since the annual surgical incidence is about 15 cases/100,000 inhabitants (DSSB, 1993) and the overall cost of echinococcosis in human and animal averaged US$ 15 million (Majorowski *et al.*, 2005). Although the definitive host is almost always a canid, a great many intraspecific variants or strains of *E. granulosus* has been described from different intermediate hosts species (Thompson & Lymbery, 1988; Eckert & Thompson, 1997). The human disease is contracted by ingestion of vegetable contaminated by eggs or by contact with a dog which may harbour different strains of *E. granulosus* (genotypes G1-sheep or G6-camel and further strains up to G10) described by Eckert & Thompson (1997) and Lavikainen *et al.* (2003). Using multilocus enzyme electrophoresis in a previous study, we demonstrated that sheep, cattle and children are infested by the same common sheep strain strain in Tunisia (Oudni *et al.*, 2004). Prevalence of hydatid cysts is low in Tunisian dromedaries (6.5 %: Lahmar *et al.*, 2004), compared to neighbouring countries such as Algeria (24.8 % to 42.1 %: Lahmar *et al.*, 2004; Dabbek, 2002), Lybia (48 %: Ibrahem & Craig, 1998), Egypt (31 %: Rahman *et al.*, 1992) or Morocco (80 %: Pandey *et al.*, 1996). It was firstly attributed to the presence of unadapted sheep strain (Lahmar *et al.*, 2004), but further studies demonstrated that it was the G6 camel strain (M'rad *et al.*, 2005) using sequences of the mitochondrial cytochrome C oxydase CO1. This difference, based on a single gene, is not useful to study population genetic structure. Single-strand conformation polymorphism (SSCP) (Gasser *et al.*, 1998; Haag *et al.*, 1999; Zhang *et al.*, 1999) have been used for the study of genetic variation between lines or strains of *E. granulosus*. The SSCP technique relies on the principle that the electrophoretic mobility of a single stranded DNA molecule in a non-denaturing gel is dependent on its struc-

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ture and size (Orita *et al.*, 1989). The technique presents the disadvantage to be extremely sensitive to the temperature variations and to be very largely dependent on the size of fragments of DNA (Kain *et al.*, 1996; Zhang *et al.*, 1999; Martins-Lopes *et al.*, 2001). Nevertheless, the addition of glycerol is a useful supplement for increasing sensitivity in SSCP acrylamid gel. The recorded variations can be interpreted in terms of population genetics (homozygotes and heterozygotes frequencies) (Haag *et al.*, 1999). In the present work, we intended to address the comparative genetic diversity and structuration of *E. granulosus* sheep and camel strains in Tunisia using SSCP.

MATERIALS AND METHODS

SAMPLES

Parasite material consisted of hepatic and pulmonary cysts from ovine and dromedary in Tunisia. 63 cysts from 41 sheep slaughtered at the abattoirs of Sousse (Centre of Tunisia) were collected. Six sheep had multiple cysts (12, 10, 7, 5, 3 and 2). We could obtain only eight cysts from eight dromedaries provided by the slaughter-house of Benguerden (South of Tunisia, where dromedaries are concentrated).

In this study, the samples analysed included isolates taken from single cyst per host animal. We considered the cyst as a sample only when different genotypes were found. We discarded the cysts sampled in a sheep, when they presented repeated genotypes, suggesting the possibility of polyembryony. Immediately after collection the protoscoleces were removed of metacestode by several washings with sterile solution of sodium chloride 0.9 % followed by sedimentation to room temperature (Babba, 1987). Then, each sediment of protoscoleces was homogenized with an equal volume of distilled water and freezed over night in liquid nitrogen.

DNA EXTRACTION AND ENZYMATIC AMPLIFICATION

After unfreezing, a centrifugation at 18,000 g for 20 min at 4° C, two volumes of lysis solution (Tris-HCl 50 mM pH 8, NaCl 100 mM, EDTA 50 mM pH 8 and SDS 1 %) were added in order to remove the protein rich supernatants and to homogenize the sediment. Total DNA of sample was extracted using a phenol/chloroform extraction (Sambrook *et al.*, 1989).

Three different targets were amplified by PCR. One target is a mitochondrial DNA region coding for the cytochrome C oxidase subunit 1 (CO1) (Bowles *et al.*, 1992a), which is used to differentiate the camel and sheep strains. Two other targets are non coding region 5' and 3' flanking regions of the gene Ag4 (a cytosolic

malate dehydrogenase) and Ag6 (a calcium-binding protein) (Haag *et al.*, 1999).

Thirty five cycles of denaturation (94° C for 30 seconds), annealing (55° C for 30 seconds) and extension (72° C for 30 seconds) were carried out. For each set of PCR reaction, negative controls (no DNA) were included. The nucleotide variation within the PCR products obtained for the three targets was screened by the SSCP method.

SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP)

A method similar to that previously described by Haag *et al.* (1998) was used. In brief, four microlitre of each PCR product were denatured before analysis by adding an equal volume of denaturing buffer (95 % formamide, 10 mM NaOH, 0.5 % bromophenol blue and 0.25 % xylene-cyanole). After denaturation at 95° C during 5 min, the samples were rapidly chilled on ice and then 3 μl of individual samples were immediately loaded onto a non denaturing 10 % (CO1 and Ag4) and 12 % (Ag6) polyacrylamide gels supplemented with glycerol. For each SSCP reaction, not denatured DNA and amplified isolates of G1 (common sheep strain: 12 sheep isolates were sequenced) and G6 genotype (camel strain, eight dromedaries were sequenced) were included (M'rad *et al.*, 2005). Electrophoresis migration was performed at 10° C for three hours (Ag4 and Ag6) to five hours (CO1) under a constant difference of potential of 200 V. The gels were stained with silver (Sambrook *et al.*, 1989), photographed and dried.

DATA ANALYSIS

Phenotypic differences in SSCP Ag4 and Ag6 banding pattern at a specific locus were used to deduce genotypes of individuals and the number of putative alleles segregating at each locus. For polymorphic loci, allelic frequencies were estimated and heterozygote frequencies expected under Hardy-Weinberg equilibrium were calculated for each population. Genetic population structure was described by the F_{st} and F_{is} indices of Wright (Wright, 1978). The F_{is} (genetic differentiation within a population) and F_{st} (genetic variation between populations), were calculated according to the method proposed by Weir & Cockerham (1984), and tested for significant deviation from 0 at the 95 % confidence level, either using boostrap (1000) or permutation (100) resamplings (Belkhir *et al.*, 2003). Positive F_{is} values indicate a departure from Hardy-Weinberg expectations toward a heterozygote deficiency, and negative values indicate an excess of heterozygotes. F_{st} values vary between 0 and 1, with 0 indicating no differentiation (random mating between individuals of the populations), and 1 complete differentiation between population. Negative values can be obtained due to cal-

culation approximations and are artefactual. Additionally, distance of Cavalli-Sforza (Cavalli-Sforza & Edwards, 1967) was used to detect differences between *E. granulosus* host origins, and differences tested by 100 permutations of individual cyst values. The CO1 gene is located in maternally transmitted mitochondrial DNA and cannot thus be the subject of a genetic interpretation in terms of homozygote and heterozygote. Thus, it was not included in the calculation of the Wright indices and the Cavalli-Sforza distance.

RESULTS

The amplification products of CO1, Ag6 and Ag4 were 460 bp, 405 bp, and 106 bp in size respectively. While there was no variation in size among the PCR products on agarose gels, SSCP analysis revealed distinct profiles amongst the different *E. granulosus* isolates. In CO1 sequences two alleles were observed (Fig. 1), one of rapid migration (C1) appearing only for the dromedary isolates and one of slow migration (C2) identical for all the sheep samples. The comparison of the SSCP pattern of our samples with those of genotype G6 (camel strain) and G1 (sheep strain) reference isolates, allows us to conclude that the C1 and C2 phenotypes corresponded to the classical camel and sheep genotypes. Four distinct SSCP Ag4 and Ag6 patterns were identified: single or double banded characteristic of homozygotes

with complementary ssDNA with the same or different electrophoretic mobility, triple or quadruple ssDNA pattern associated to several dsDNA bands and characteristic of heterozygote individuals (Figs 2, 3). Allelic frequencies, observed and expected heterozygote frequencies for each population are shown in Table I. The difference observed between the dromedary samples and the isolates originating from sheep showed significant F_{st} values ($F_{st} = 0.46$, $p < 0.05$ using 100 permutations) or Cavalli-Sforza distance (0.62, $p < 0.05$ using 100 permutations). There was an apparent deficiency (Ag4) or excess (Ag6) of heterozygotes in the sheep strain (see Table I). The global F_{is} (0.20, bootstrap confidence interval 95 %: - 0.13 to 0.70) was indicative of Hardy-Weinberg equilibrium. The sheep strain Ag4 F_{is} was 0.38 (confidence interval 95 %: - 0.14 to 0.67) which indicates equilibrium to Hardy-Weinberg. The sheep strain Ag6 F_{is} was - 0.23 (confidence interval 95 %: - 0.37 to - 0.03), showing a slight excess of heterozygotes. The F_{is} of the camel strain was 1 for Ag4 and Ag6, which demonstrates a deficiency in heterozygotes.

DISCUSSION

To date, few studies using PCR-SSCP approaches in *E. granulosus* were carried out (Haag *et al.*, 1998; Haag *et al.*, 1999; Zhang *et al.*,1999; Kamenetzky *et al.*, 2002). The technique presents the disad-
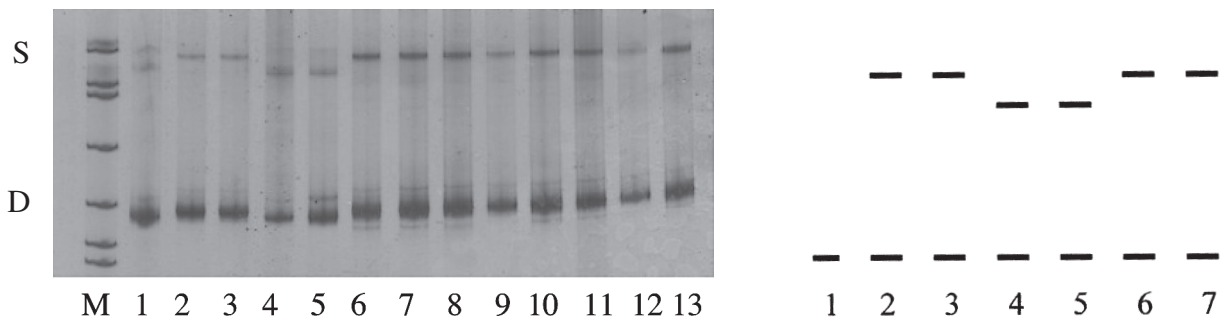

Fig. 1. – SSCP analyses of mitochondrial cytochrome C oxydase 1 (CO1). S: single-stranded DNA, D: double-stranded DNA, M: pGEM DNA marker, (Promega), lane 1: not denatured DNA, lanes 2, 3, 6 to 13: C2 phenotype, lanes 4 and 5: C1 phenotype.
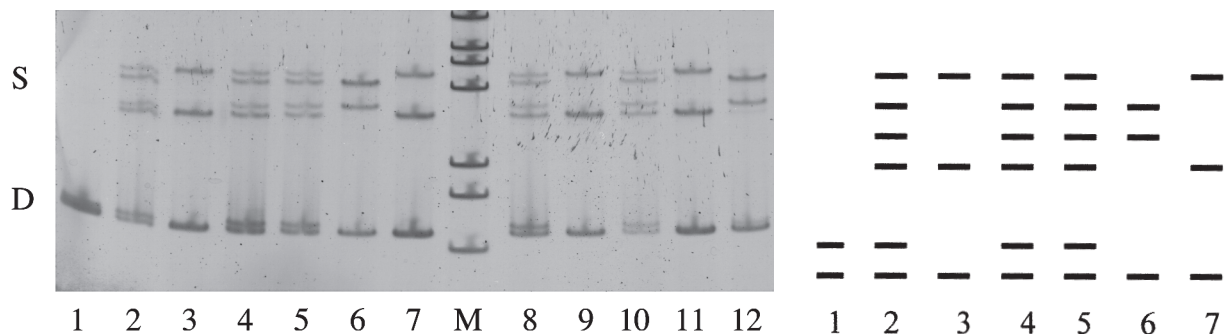

Fig. 2. – SSCP analyses of Ag4 DNA sequence. S: single-stranded DNA, D: double-stranded DNA, M: pGEM DNA marker, (Promega), lane 1: not denatured DNA, lanes 2, 4, 5, 8, 10: Heterozygotes A1A3, lanes 3, 7, 9, 11: Homozygotes A1A1, lanes 6 and 12: Homozygotes A3A3
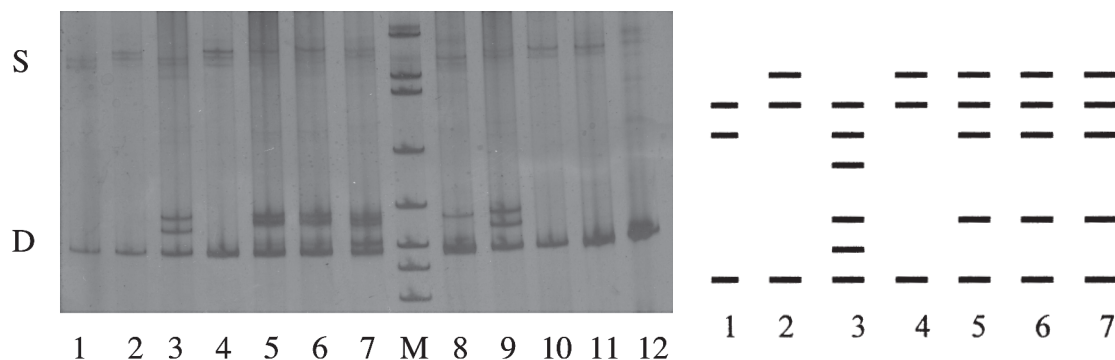
Fig. 3. – SSCP analyses of Ag6 DNA sequence. S: single-stranded DNA, D: double-stranded DNA, M: pGEM DNA marker, (Promega), lanes 1 and 8: homozygotes a1a1, lanes 2, 4, 10, 11: homozygotes a3a3, lanes 3 and 9: heterozygotes a1a2, lanes 5, 6, 7: heterozygote a1a3, lane 12 : not denatured DNA.

Locus	Allele	Sheep isolates (41)	Dromedary isolates (8)
Ag4	A1	0.84	0.25
	A2	0.12	0.00
	A3	0.04	0.00
	A4	0.00	0.75
Hexp.		0.28	0.37
Hobs.		0.17	0.00
Ag6	a1	0.57	0.25
	a2	0.30	0.00
	a3	0.13	0.75
Hexp.		0.56	0.40
Hobs.		0.71	0.12
CO1	C1	0	1
	C2	1	0

Hexp.: Expected heterozygote frequency calculated on the basis of Hardy-Weinberg equilibrium.
Hobs.: Observed heterozygote frequency.

Table I. – Sheep and dromedary *E. granulosus* allelic frequencies at the Ag4, Ag6 and CO1 loci.

vantage to be extremely sensitive to the temperature variations and to be very largely dependent on the size of fragments of DNA (between 100 and 500 bp) (Kain *et al.*, 1996; Zhang *et al.*,1999; Martins-Lopes *et al.*, 2001). The present study demonstrates that PCR-linked SSCP provides a method to display variation between *E. granulosus* strains, which has significant implications for studying the population genetics of this parasite and for epidemiology and disease control. As it was previously described by Haag *et al.* (1999) in sheep strain, the most polymorphic loci found were Ag4 with four alleles, followed by Ag6 with three alleles. The dromedary prevalence of hydatidosis in Tunisia is low (6,5 %: Lahmar *et al.*, 2004) and consequently our samples from dromedary were few which makes comparison with sheep strain tentative. The dromedary isolates were characterized, when compared to sheep samples, by the absence of alleles A2 and A3, and the presence of the A4 (Ag4 gene), also found by Kamenetzky *et al.* (2002), and classical C1 (CO1 gene) alleles. The F_{st} (0.46) between sheep and dromedary isolates is relatively high compared to 0.07 between sheep and cattle samples of the same sheep strain using isoenzymes (Oudni *et al.*, 2004).

In sheep isolates no departure from the Hardy-Weinberg equilibrium was detected whereas a strong deviation was found in the camel isolates. The deficiency in heterozygote in camel isolate could possibly be explained by a Wahlund effect as described in Richardson *et al.* (1986). The Wahlund effect is an apparent deficiency in heterozygote due to the mixing of populations with different allelic frequencies. This may arise because it was impossible to have any information on the precise origin of the slaughtered animals, and so we considered each host as originating from different area. The low genetic variability in the camel strain could be explained by a founder effect (few worms introduced into a small area and submitted to special climatic conditions) which induced random genetic drift and fixation alleles. This low genetic diversity could also be due to the small size of our dromedary sample. Another explanation of this deficiency of heterozygotes is due to the mode of reproduction of *E. granulosus* based on an important self-fertilisation as presented by Haag *et al.*, 1998. Selfing tends to increase the homozygote genotypic frequencies at the expense of the heterozygote genotypes. A positive F_{is} was recorded in sheep isolates using isoenzymes (Oudni *et al.*, 2004) but was not found in the present investigation. This discrepancy could be due to the existence of null alleles when interpreting isoenzyme (Oudni *et al.*, 2004) or SSCP (Haag *et al.*, 1998) data, or more probably to a lack of statistical power due to the reduced number of loci and samples used in our Ag4 / Ag6 study. Selfing is a cause of deficiency in heterozygotes but it was found only in camel strain on a very limited number of isolates and it needs further evaluations.

Mémoire

A more extensive study should be performed in the dromedary breeding areas of Tunisia, as camel strain could be a reservoir for human infection, as already shown for example in Argentina (Guarnera *et al.*, 2004), in Nepal (Zhang *et al.*, 2000), in Iran (Harandi *et al.*, 2002), in Kenya (Dinkel *et al.*, 2004) and in Mauritania (Bardonnet *et al.*, 2002).

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